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Review

# TRPML and lysosomal function

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**Abstract**

Mucolipin 1 (MLN1), also known as TRPML1, is a member of the mucolipin family. The mucolipins are the only lysosomal proteins within the TRP superfamily. Mutations in the gene coding for TRPML1 result in a lysosomal storage disorder (LSD). This review summarizes the current knowledge related to this protein and the rest of the mucolipin family.


*Keywords:* Lysosomal storage disorder; Mucolipin protein family; Endocytosis and lysosomal function; Mucolipidosis

## 1. Introduction

TRPMLs are encoded by the MCOLN genes. TRPML1 is encoded by MCOLN1 localized in humans at chromosome 19p13.2–13.3 while the 2 other genes—MCOLN2 and MCOLN3 are localized at chromosome 1p22.3 encoding TRPML2 and TRPML3 respectively. MCOLN1 is mutated in humans in a lysosomal storage disorder—Mucolipidosis type IV (MLIV) (MIM #252650), an autosomal recessive, neurodegenerative disease characterized by severe psychomotor retardation and vision impairment due to retinal degeneration and cornea opacity (Reviewed by [1–3]). Achlorhydria and iron deficiency are also part of the clinical symptoms [4,5]. Unlike most other lysosomal disorders MLIV progresses extremely slowly, thus patient ages range from 1 year to the late thirties and forties and life expectancy is not clear yet. Milder patients, particularly in the psychomotor impairment, were also reported [6–8].

MLIV is found in increased frequency among Ashkenazi Jews (AJ) with a heterozygote frequency of 1:100 [9,10]. Over 15 mutations were identified in MLIV patients, Jews and non-Jews, including two MCOLN1 founder mutations in the AJ population comprising 95% of the mutated alleles among AJ MLIV patients [3]. These founder mutations date approximately 40 generation ago [11].

The identification of MCOLN1 mutations opened the door for accurate patient diagnosis as well as prenatal diagnosis and heterozygote identification. Lately MLIV is included in the population screening program operated in various countries with high concentrations of AJ in an attempt to ascertain high risk couples, in which both parents are heterozygotes and offer them options for family planning (prenatal diagnosis, marriage decisions), as a preventative program to reduce the number of newly born MLIV patients in the high risk population [12].

### 1.1. Lysosomal storage

MLIV is classified as a mucolipidosis due to the simultaneous lysosomal accumulation of lipids together with water soluble substances [13]. This characteristic heterogeneous storage is observed in cells of every tissue and organ of MLIV patients and principally best demonstrated by electron microscopy [14–18]. The storage material in MLIV is autofluorescent [19]. This typical finding is also present in cultured amniotic fluid cells obtained from MLIV affected fetuses and thus this technique was used for prenatal diagnosis purposes in the era preceding the gene discovery [20,21]. Chemical analysis led to the identification of the stored substances as a variety of lipids namely, gangliosides, phospholipids and neutral lipids while the water soluble materials included primarily mucopolysaccharides and glyco-

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proteins [2,3]. Histochemical analysis indicated that gangliosides were predominately stored in neural tissues while phospholipids were mostly accumulating in visceral organs [22]. It should be noted that despite the early onset of the disease in early infancy and the clear evidence of the characteristic lysosomal storage in these early ages, the fact that the disease remains in an apparent steady state or very slow deterioration for at least 2–3 decades may suggest that the storage is not as massive and progressive as is found in most other lysosomal storage disorders.

What leads to the storage in MLIV? Earlier studies indicated that the heterogeneous storage does not stem from the impairment or deficiency of a lysosomal hydrolase that could account for this phenomenon [23]. Studies in cultured fibroblasts with radiolabeled compounds such as phosphatidylcholine or gangliosides, which constitute the bulk of the storage in MLIV, or with fluorescent lipid analogs, indicated that the abnormal accumulation results from a defect in the late stages of the endocytosis process of membrane compounds leading to the impairment of lysosomal biogenesis or, alternatively, a defect in the normal maintenance of the mature lysosomes [23–26]. It should be pointed out that another LSD, namely, Niemann–Pick disease type C (NPC) (MIM #257220) [27] is also suggested to be involved in defective vesicular trafficking of late endosomes, although with different cellular and biochemical characteristics [28–30], thus, these two disorders (MLIV and NPC) constitute a distinct group within the LSD of endosomal/lysosomal trafficking impairment leading to defective lysosomal biogenesis or function.

Catabolism of the accumulated materials in MLIV still does occur in the storage vacuoles (early lysosomes? Multivesicular bodies?) of MLIV patients, though in an altered rate and pattern (for instance; degradation products are not exported normally from these organelles to the Golgi apparatus) [24,26]. This helps to explain the clinical manifestations of this disease, as outlined above; since despite the early onset of the disease and severe neurological involvement, MLIV patients remain in an apparent steady state for decades. This may indicate that indeed the slower catabolism rate prevents a massive storage as is the case in most other LSD, and hence, the extremely slow progression of the clinical picture in these patients.

### *1.2. Genetics*

The gene MCOLN1 was identified by linkage studies [31–33] following its mapping to human chromosome 19p13.2–13.3 [34]. The identification of this gene triggered the identification of two other homologous genes, namely, MCOLN2 and MCOLN3, which mapped to chromosome 1p22.3. The MCOLN genes code for TRPML proteins that show about 60% amino acid homology to each other. The identification of the human genes lead to the identification of similar genes in mice, *C. elegans* and *Drosophila*. Over 15 different MCOLN1 mutations have been identified in MLIV patients, including, partial gene deletions, insertions, splicing, missense and nonsense mutations [3]. It should be noted that most missense mutations are located in the putative transmembrane domains. The clinical severity among the various patients is very similar regardless the nature of the mutations; but one in-frame deletion mutation of amino-acid No. 408—phenylalanine, is found in a mild patient in compound heterozygosity with a splicing mutation [3]. Except for AJ, MLIV is considered to be rare in other populations.

Unlike MCOLN1 there are no reports associating MCOLN2 or MCOLN3 with human diseases. Nevertheless, spontaneous mutations in MCOLN3 in Varitint–Waddler mice were shown to be associated with early deafness, vestibula defects, pigmentation abnormality and perinatal lethality [35]. At present no human counterpart was reported. Mutations in a mucolipin-like gene in *C. elegans* — CUP5 was reported to be associated with general lysosomal storage phenomena of a similar nature as is present in cells of MLIV patients, as well as increased cell death leading to embryonic lethality [36,37]. Recent reports have linked the embryonic lethality in CUP5-null *C. elegans* to starvation of embryonic cells and general developmental defects [38]. These observations were suggested to stem from defective endocytosis of nutrients and the general accumulation of a genetic suppressor, the ATP-binding cassette (ABC) transporter MRP-4, in CUP5-null cells [38,39].

Abnormal mitochondrial function due to increased mitochondrial fragmentation is observed in MLIV cultured fibroblasts, as well as in cell lines from other lysosomal storage disorders, as a result of inefficient autophagolysosomal recycling of mitochondria [40]. This leads to decreased $Ca^{2+}$ buffering capacity in MLIV cells which increases susceptibility to a caspase-8-dependent apoptotic pathway. This phenomenon might also explain the increased sensitivity to apoptosis in *C. elegans* as described above. The functional link between mutations in the MCOLN1 gene, mitochondrial aberrations and the clinical manifestations in MLIV still remains to be seen.

### *1.3. Mucolipins and subcellular localization*

Three proteins are encoded by the MCOLN genes, namely, TRPML1, TRPML2, and TRPML3 also referred as MLN1–3 or Mucolipin 1–3, respectively. Following the identification of the relevant genes the putative structure of the protein products was deduced by computerized analysis [31]. This indicated that TRPML1 is 580 amino acids long consisting of 6 transmembrane domains which attribute this protein to the TRP superfamily [41,42], although it does not contain ankirin domains. The two other TRPML proteins have similar structure.

The physiological function of the mucolipins has not yet been fully elucidated, but electrophysiological analyses indicate that they function as cation channels. This was particularly demonstrated in TRPML1 channels. Based on previous findings (see above) it is expected that TRPML1 function would be related to the biogenesis of lysosomes or their normal maintenance. Indeed, studies with expression vectors and immunohistochemistry demonstrated that TRPML1 is located to lysosomal vacuoles [26,43–47].

Site directed mutagenesis indicated the role of two dileucine motifs, one at the N-terminal and the second at the C-terminal tail of TRPML1, as subcellular targeting motives. Experiments

with a C-terminal construct of TRPML1 (consisting of amino acids 518–580 fused to the C-terminus of Tac) identified a C-terminal dileucine motif (amino acids 573–578: EEHSLL) that represents an AP2-dependent internalization signal responsible for targeting TRPML1 to early endosomes from the plasma membrane [44]. In addition, it was found that palmitoylation of cysteines in close proximity to the C-terminal dileucine motif (on amino acids 565–567) regulates this endocytotic signaling. However, the full length TRPML1 protein proved to be independent of this targeting signal since removal of the C-terminal dileucine motif was insufficient to mistarget the full length protein from its proper lysosomal subcellular localization [26,44–47]. As such, another dileucine motif in the N-terminus of TRPML1 (consisting of amino acids 11–16: ETERLL) was identified, containing sufficient lysosomal targeting information [44,47]. Miedel et al. [45] characterized the trafficking of TRPML1 by reporting a strict AP1 dependence for the lysosomal localization of the full length protein [45]. Thus, taken together, it appears that the N-terminal dileucine motif is the primary targeting signal of TRPML1 and it is apparently also AP1 dependent. A simple AP1 knock-down experiment with a Tac-TRPML1 N-terminus construct should clarify this point. Furthermore, it has recently been reported in human fibroblasts that TRPML1 may play a role in lysosomal exocytosis [48]. This process is characterized by fusion of lysosomes with the plasma membrane whereby lysosomal contents are exocytosed. In such a case, the normally lysosomal TRPML1 may find itself in the "foreign territory" of the plasma membrane. Hence, the AP2-dependent C-terminal dileucine internalization motif may be of critical importance to retrieve TRPML1 to lysosomes from the plasma membrane following such an exocytotic event.

The cellular localization of TRPML2 and TRPML3 is less clear, but a recent study which is confirmed in the present report, indicates that these proteins together with TRPML1 are capable of interacting with each other (Fig. 3 and [47]). While TRPML1 and TRPML2 can each transport independently from the Golgi to lysosomes, TRPML3 requires one of the other two for its lysosomal localization. These data suggest that in fact all the members of the mucolipin family are lysosomal proteins but this should await further confirmation and characterization. Nevertheless, these observations imply that transfection studies leading to overexpression of TRPML3, unlike the other two TRPML proteins, will not reflect the natural localization of this protein as it will preclude stoichiometric association of the excess TRPML3 with the other TRPML members thereby diverting it from targeting to lysosomes.

The fate of TRPML1 in MLIV patients depends upon the nature of the mutations in the MCOLN1 gene. Naturally, gross genetic alterations, such as partial gene deletions or splicing mutations (both of which are the AJ founder mutations), as well as insertions or non-sense mutations, will basically lead to the absence of any functional protein. On the other hand, several missense mutations were shown to cause the misfolded protein to be retained in the endoplasmic reticulum, apparently due to the chaperon "proof reading" system, thereby preventing these mutant proteins from reaching the lysosome [43,46]. Other missense mutations do reach the lysosomes, but have been shown to affect TRPML1 channel activity. Table 1 summarizes the published data regarding the fate of some of the TRPML1 missense mutations. It should be noted, however, that with the exception of the mild F408 in-frame deletion, all MLIV mutations to date cause the same, or similar, severe clinical manifestations.

### *1.4. Mucolipin structure and protein interactions*

The putative structure of TRPML1 is pictured in Fig. 1. TRPML1 is palmitoylated on its C terminus at residues 565–567 [44] (and unpublished results). The suggested function of this palmitoylation has been described above with regard to trafficking of the protein.

In addition, TRPML1 is also a glycoprotein with four putative N-linked glycosylation sites (at amino acid positions 159, 179, 220, and 230) in its long luminal loop between transmembrane domains 1 and 2. TRPML1's N-linked sugars are sialylated, at least in part, in the Golgi prior to the protein's transport to lysosomes [45,46]. Interestingly, the N-glycosylation sites have been used to map a physical and temporal pattern of TRPML1 cleavage in its long luminal loop. TRPML1 is cleaved into two distinct cleavage products resulting from a directed cleavage of TRPML1 between two glycosylation sites at amino acid positions 179 and 220, respectively [45]. Kiselyov et al. [46] further characterized the cleavage site by performing N-terminal sequencing on the C-terminal cleavage product yielding a putative cleavage site just upstream of the proline at amino acid position 201. Both studies observed that the N-linked sugars on both the N and C-terminal cleavage products are sialylated, suggesting that the temporal cleavage of TRPML1 occurs post-ER, in a prelysosomal compartment. In addition, both studies also reported that each cleavage product co-immunoprecipitates the other, suggesting that the cleavage products may remain associated with each other after cleavage. However, one very important discrepancy was observed between these two studies as Kiselyov et al. [46] were able to inhibit cleavage of TRPML1 with cathepsin B protease inhibitors while Meidel et al. [45] resorted to the broad

Table 1
Wild type MCOLN1 and mutations

| MCOLN1 mutation | Localization | Channel activity | Low pH inhibits channel activity | High $Ca^2$ inhibits channel activity | Cleavage | References |
|---|---|---|---|---|---|---|
| Wild type | Lysosome | Yes | Yes | Yes | Yes | [26,43–47, 59,60] |
| T232P | ER | Slight | ND[a] | ND | No | [43,46] |
| D362Y | ER | No | ND | ND | No | [46,60] |
| F408del | Lysosome | Yes | No | No | Yes | [43,59,60][b] |
| V446L | ER[c] | Yes | No | No | ND | [26][c], [59,60] |
| F465L | Lysosome | No | ND | ND | Yes | [26,43,46][b] |

[a] No data.
[b] Zeevi, D.A., Frumkin, A, and Bach, G., unpublished data.
[c] Authors reported unpublished data.

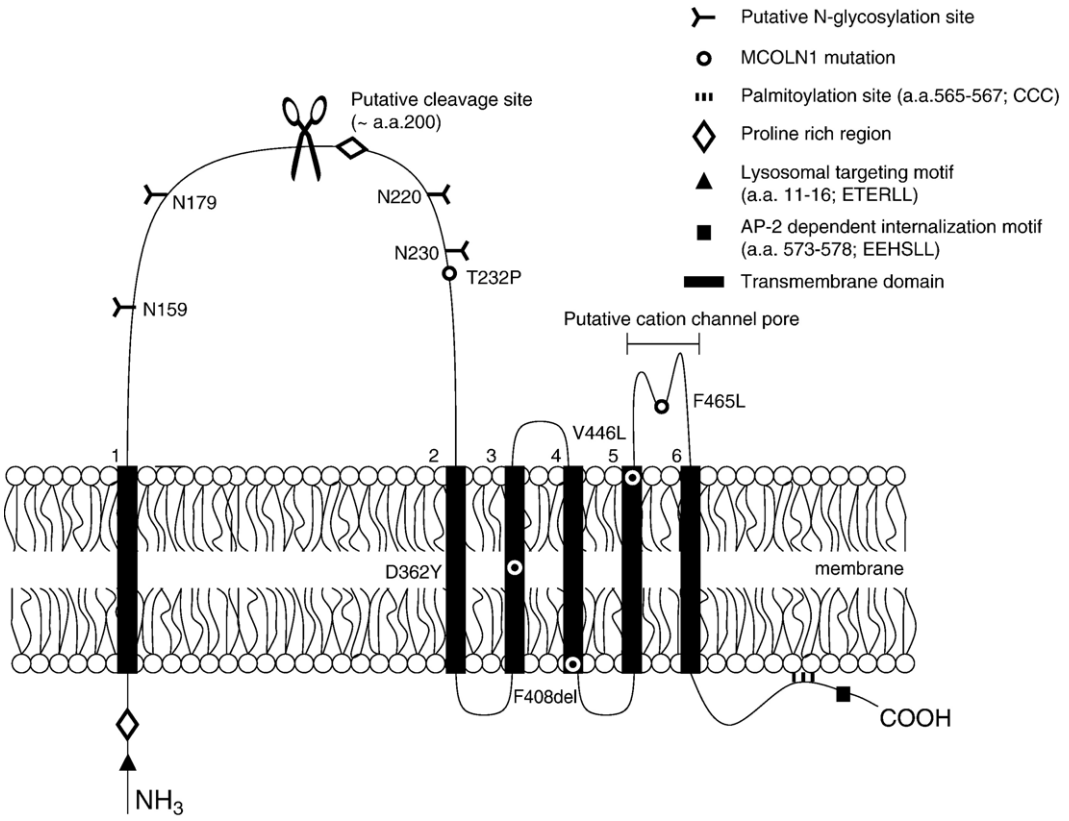


Fig. 1. The putative structure of TRPML1.

lysosomal serine, plasmin, and cysteine protease inhibitor, leupeptin, to prevent cleavage. Nonetheless, both studies did qualify their results by not ruling out the possibility of a number of different proteases being responsible for the cleavage of the protein.

The most significant finding, with regard to TRPML1 cleavage, is that the endogenous protein has also been found to undergo cleavage. In addition, it has also been found that the N and C-terminal cleavage products are in excess with relation to the full length protein in HEK 293, human skin fibroblast, and bovine brain cells ([46] and Fig. 2). This suggests that TRPML1 cleavage may play an important role in regulating the function of the protein. It has already been proposed that cleavage inactivates the channel function of TRPML1 [46].

Overexpression of TRPML1 has led to the discovery of two predominant observations. The first is the distinct possibility of plasma membrane localization predominating over the widely agreed upon lysosomal localization of the protein. This phenomenon was used in HEK 293 cells where plasma membrane localization facilitates simple electrophysiological recordings via standard patch-clamp methods [46,49]. Nonetheless, all such electrophysiological recordings must be qualified in light of the fact that the lysosomal membrane is different, topologically and structurally, from the plasma membrane. Aside from both membranes featuring different populations of glycolipids, sphingolipids, and phospholipids; the lysosomal membrane is also a specialized single lipid bilayer membrane as opposed to the double lipid bilayer plasma membrane [50–52]. These and other differences between the lysosomal membrane and the plasma membrane likely reflect important influences upon electrophysiological recordings.

The second observation that predominates in TRPML1 but also TRPML2, and TRPML3 overexpression, is the formation of indissociable aggregates. These aggregates have appeared in pictured immunoblots of TRPML1 in a number of different publications [43–45,53]. We also observed a similar observation with regard to TRPML2 and TRPML3 (Fig. 3). While

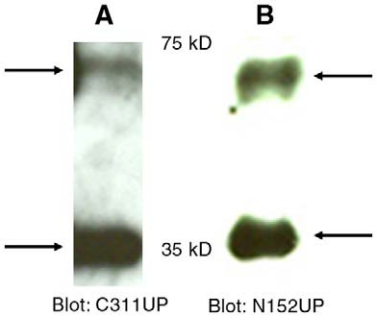


Fig. 2. Endogenous TRPML1. (A) An extract from HEK 293 cells was probed with C311UP following 12% SDS-PAGE. The full length endogenous TRPML1 protein (65 kDa) and the C-terminus cleavage product (~35–40 kDa) are demarcated with arrows. (B) An extract from HEK 293 cells was probed with N152UP following 12% SDS-PAGE. The full length endogenous TRPML1 protein (65 kDa) and the N-terminus cleavage product (~35–40 kDa) are demarcated with arrows. These data confirm that the endogenous TRPML1 protein is also cleaved in vivo as described elsewhere [46].

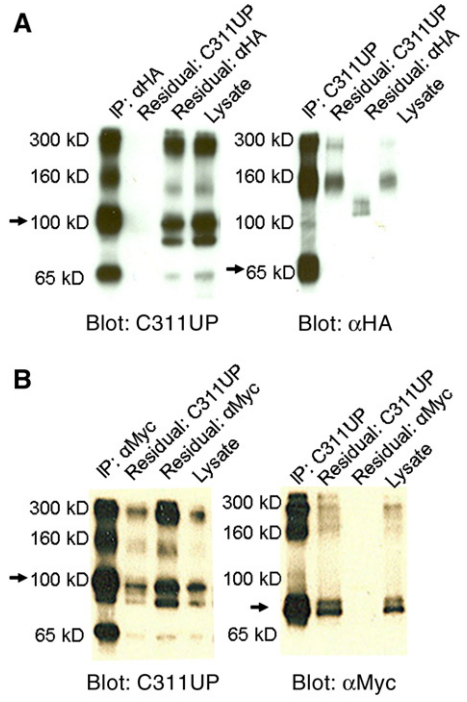


Fig. 3. TRPML1 co-immunoprecipitates TRPML2 and TRPML3. C311UP was used to immunoprecipitate (IP) and immunoblot (Blot) TRPML1-YFP, monoclonal anti-HA antibody was used to immunoprecipitate and immunoblot TRPML2-HA, and monoclonal anti-c-myc antibody was used to immunoprecipitate and immunoblot c-Myc-TRPML3. (A) TRPML2-HA co-immunoprecipitates TRPML1-YFP (left panel) and TRPML1-YFP co-immunoprecipitates TRPML2-HA (right panel). "Residual" lanes represent unbound protein following immunoprecipitation with the indicated antibody. "Lysate" lanes represent cell lysates not treated with antibodies. Note the upper bands on both immunoblots representing aggregated forms of these proteins. The monomeric bands are demarcated with arrows. (B) c-Myc-TRPML3 co-immunoprecipitates TRPML1-YFP (left panel) and TRPML1-YFP co-immunoprecipitates c-Myc-TRPML3 (right panel). Note the upper bands on both immunoblots representing aggregated forms of these proteins. The monomeric bands are demarcated with arrows.

endogenous expression of TRPML2 and TRPML3 cannot be verified due to the lack of antibodies that recognize these endogenous proteins, characterization of the endogenous TRPML1 has revealed no indissociable aggregates in immunoblots carried out on control HEK 293 cells (Fig. 2). It is assumed that due to the highly hydrophobic nature of the TRPML integral membrane proteins, aggregation in overexpression would be unavoidable.

Nevertheless, overexpressed protein aggregation does not preclude the possibility of protein–protein interactions existing between the different TRPMLs and each other. One common feature of many TRP proteins is the ability to homo- and hetero-multimerize with closely related members of the same subfamily [54,55]. Indeed, Venkatachalam et al. [47] reported interactions between TRPML1 and both TRPML2 and TRPML3, respectively, using a FRET-based approach. This same approach demonstrated an interaction between TRPML2 and TRPML3 as well. Here we further report that TRPML1 also co-immunoprecipitates TRPML2 and TRPML3 and vice-versa (Fig. 3). These protein interactions and others await verification with controlled experiments on the endogenous TRPML proteins in the future. Nonetheless, the concept of TRPML1 being part of a complex is important when considering optimization of future experiments to characterize the structure and function of the protein. The concept of a complex structure involving the different mucolipins and the implications for MLIV awaits further elucidation.

## 2. TRPML1 channel activity

The mechanism which accounts for the pathogenesis of MLIV is not clear. The initial assessment of the function of TRPML1 was suggested by its predicted protein structure [31], which indicated one transmembrane (TM) helix in the amino-terminal region and at least five transmembrane domains in the carboxy-terminal half of the protein (Fig. 1). The putative structure of six transmembrane domains along with a TRP cation channel motif spanning the third to the sixth TM domains suggested that the protein belongs to the TRP superfamily [56] with a high resemblance to the polycystin-2/TRPP2 protein [57]. Thus it was expected that TRPML1 function as a cation channel.

The ability to perform electrophysiological analyses of channel activity, in order to prove the above assumption, suffers from serious limitation since the protein is located in the lysosomal and/or late endosomal membrane. Therefore electrophysiological recordings have been made possible only after overexpressing the protein in different cell types in ectopic locations.

Overexpression of the protein to the *Xenopus* oocyte plasma membrane observed TRPML1 as a novel channel with a large conductance and permeability to $Na^+$, $K^+$ and $Ca^{2+}$ [58]. An overexpressed protein which is located in an ectopic location does not necessarily reflect the endogenous activity of the channel, especially in an environment where other channel proteins might interact with the overexpressed protein. Thus, a different approach to record the channel activity of the overexpressed protein in a relatively isolated environment was taken [59]. The channel activity was assessed in endosomal vesicles of MLIV patient and in TRPML1 over-expressing cells, as well as in proteoliposomes which contained the in vitro translated protein [60]. Electrical recordings of the channel from both preparations indicated that wild type TRPML1 is a multiple subconductance non-selective cation channel whose function is inhibited by $Ca^{2+}$ transport and a reduction of pH [59,60]. Since it was shown in this system that mutated channels lost their ability to be regulated by $Ca^{2+}$ and by lowering pH, it was suggested that this might hint to the mechanism which underlies the pathophysiology of the disease. Intravesicular pH is critical in endosomal/lysosomal fusion, suggesting that its regulation (possibly by $Ca^{2+}$) is essential for the ability of vesicular organelles to fuse [61,62].

In a different approach, the channel conductance was measured by whole cell current recording after mistargeting the protein to the plasma membrane in HEK 293 cells. In this system TRPML1 showed characteristic strong outwardly rectifying current [46]. Following these recordings a new mechanism was suggested for the pathogenesis of MLIV.

Soyombo et al. [49] indicated that TRPML1 in fact functions as a proton channel and demonstrated a decrease in the lysosomal pH in cultured cells of MCOLN1 mutants when compared to normal. This suggests that TRPML1 might function as a pH regulator of lysosomes, whose function is to pump $H^+$ ions out of these organelles when this milieu becomes acidified during the catabolism process. Thus, TRPML1 functions as a pH regulator in maintaining the required acidity for the normal activity of lysosomal hydrolases. It was also demonstrated that supplying lysosomes with weak bases lead to the correction of lysosomal storage as judged by electron microscopy observations. These data remain to be confirmed and further characterized.

The most recent study of TRPML1 channel activity claims that none of the above electrophysiological recordings could be reproduced [26]. This study proves that correct targeting of TRPML1 to lysosomes and the integrity of its predicted ion pore are essential for its physiological function. The authors mention the following reasons to explain the difficulty of obtaining electrophysiological data from TRPML1 in transfected cells: (1) the channel may be inactivated by proteolytic cleavage; (2) subunits which might be needed for channel oligomerization may be missing; and (3) possible TRPML1-interacting proteins, which could be needed for gating the channel, may also be missing.

MLIV research has presented with conflicting results well before the cloning of the MCOLN1 gene. Nowadays, the conflicting electrophysiological recordings of the overexpressed protein are confounded by confusing data with regard to lysosomal pH of MLIV patient fibroblasts. While we found elevated pH [63], others reported normal [26,64] or reduced pH [49]. While the pH discrepancies could be explained by the autoflourescence of the MLIV fibroblasts, the other conflicting results could be explained by the artificial conditions of the mistargted and overexpressed proteins. In their review which deals with “Cellular and molecular function of mucolipins and polycystin 2”, Qian and Noben-Trauth [65] mention a variety of experiments with polycystin 2 that describe its localization at various intracellular locations. They suggest that these discrepancies could be explained by differing methodologies, however, they also predict that differing results may stem from dynamic intracellular trafficking of polycystin-2 and that this trafficking may display variable regulation in various tissues. These principles might hold for TRPML1, the closest relative of polycystin 2, but only further investigation into the dynamic trafficking of this protein would give a firm support to this idea.

## Acknowledgment

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## Appendix A. Materials and methods

### *A.1. TRPML Expression Constructs*

TRPML1-YFP was prepared by cloning MCOLN1 cDNA (Genbank Accession no.: AF249319) derived from control human skin fibroblasts into the pEYFP-N1 vector (Clontech) with *Nhe*I and *Bam*HI restriction enzymes. This construct fused YFP to the C-terminus of TRPML1. Myc-TRPML3 was prepared by cloning the MCOLN3 CDS (Genbank Accession no.: NM_018298) derived from control human lymphoblastoid cells into the pCS2+MT vector using *Eco*RI and *Xba*I restriction enzymes. This construct fused six Myc tags to the N-terminus of TRPML3. TRPML2-HA was prepared by removing the Myc tags from pCS2+MT with *Bam*HI and *Xho*I restriction enzymes. The MCOLN2 CDS (Genbank Accession no.:BC104893), derived from control human lymphoblastoid cells, was then cloned into the *Bam*HI and *Xho*I restriction sites with an HA-coding tag (sequence: ATGTACCCATACGATGTTCCAGATTACGCTTAA) appended to the 3′ end of the CDS. All inserts orientation and polymerase fidelity were verified by restriction enzyme mapping and sequencing.

### *A.2. Cell culture and transient transfection*

HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml penicillin/streptomycin. Transient co-transfection was carried out in 100-mm dishes with 3 μg of total plasmid DNA (1.5 μg of each plasmid), and 9 μl of FuGENE-6 (Roche Molecular Biochemicals).

### *A.3. Anti-TRPML1 antibodies*

Glutathione-S-Transferase (GST) fusion proteins were generated by cloning each of the N and C terminal portions of TRPML1 (corresponding to amino acids 1–70 and 520–576, respectively) into the bacterial expression vector, PGEX-3X (Amersham BioSciences), with *Bam*HI and *Eco*RI restriction enzymes. These constructs were used to express the N and C terminal portions of TRPML1 at the carboxyl end of GST in BL21 bacteria. Each GST fusion protein was subsequently purified from bacterial lysates with glutathione–agarose (Amersham BioSciences) and used to immunize New Zealand White rabbits to generate polyclonal antisera. Antisera N152 was raised against the GST-TRPML1 N terminus fusion protein and antisera C311 was raised against the GST-TRPML1 C terminus fusion protein.

Affinity purified antibodies were prepared by synthesizing small peptides located within the N152 and C311 epitopes on TRPML1. Peptide NH-CPTPPEEEDLRRRL-COOH, corresponding to TRPML1 amino acids 33–45 with an appended N terminus cysteine, was used to purify N152; and peptide NH-CQDSPTSGKFRRGSG-COOH, corresponding to TRPML1 amino acids 544–556, was used to purify C311. These peptides were each coupled via their N terminus cysteines to SulfoLink Coupling Gel (Pierce Biotechnology) followed by affinity purification of the corresponding antisera. The resulting affinity purified antibodies were called N152UP and C311UP, respectively. The specificity of these antibodies was verified by recognition of the transfected TRPML1-YFP protein (as verified by mass spectrometry analysis), by blocking the signal with the peptides used to affinity purify the specific antibodies

and by the absence of a specific signal in TRPML1-null lymphoblastoid cell extracts.

### A.4. Immunoblotting

Cells were solubilized in lysis buffer containing 1% Triton X-100, 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and complete TM protease inhibitor cocktail (Santa Cruz). Lysates were diluted in Laemmli sample buffer and heated to 55 °C prior to SDS-PAGE and immunoblotting with monoclonal anti-HA (HA-7, Sigma), monoclonal anti-c-Myc (9E10, Santa Cruz) or anti-TRPML1 antibodies, C311UP/N152UP. Blots were developed with Western Blotting Luminol Reagent (Santa Cruz) unless endogenous TRPML1 proteins were probed, in which case blots were developed with Lumiglo Reserve Enhanced Chemiluminescence (KPL).

### A.5. Immunoprecipitation

Co-transfected HEK 293 lysates were divided into two separate tubes and immunoprecipitated with monoclonal anti-HA, monoclonal anti-c-Myc, or C311UP antibodies, as necessary. Immunocomplexes were recovered at 4 °C overnight with Protein A/G PLUS-agarose (Santa Cruz). The unbound protein solution was saved for immunoblotting and the immunoprecipitate was then washed five times with lysis buffer followed by a final wash in 50 mM Tris pH 8. Precipitated proteins were eluted with 2× Laemmli sample buffer and heated to 55 °C for 5 min prior to 10% SDS-PAGE and immunoblotting as described above. Prior to co-immunoprecipitation experiments, it was determined that C311UP does not cross-react with TRPML2 and TRPML3.

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